Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/EP05/002374

International filing date: 04 March 2005 (04.03.2005)

Document type: Certified copy of priority document

Document details: Country/Office: EP
Number: 04077656.9
Filing date: 27 September 2004 (27.09.2004)

Date of receipt at the International Bureau: 12 May 2005 (12.05.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

PCT/EP 05 / 02374

Europäisches Patentamt | European Patent Office | Office européen des brevets

Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

04077656.9

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

Europäisches Patentamt
European Patent Office
Office européen des brevets

Bescheinigung Certificate Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

04077656.9

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

Anmeldung Nr:
Application no.: 04077656.9
Demande no:

Anmeldetag:
Date of filing: 27.09.04
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

DSM IP Assets B.V.
Het Overloon 1
6411 TE Heerlen
PAYS-BAS

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Process for cell culturing by continuous perfusion culturing of cells

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12N5/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PL PT RO SE SI SK TR LI

PROCESS FOR CELL CULTURING BY CONTINUOUS PERFUSION CULTURING OF CELLS

The invention relates to a process for the culturing of cells by continuous perfusion culturing of a cell culture comprising cell culture medium and cells, wherein cell culture medium is added to the cell culture and wherein the cell culture is circulated over a cell retention device resulting in an outflow of liquid comprising less cells than the cell culture.

Such processes are known in the art and are known as perfusion processes. A disadvantage of said processes is that some cells form aggregates. This is especially troublesome if they form aggregates of 5 cells or more and when the aggregates comprise in total 5 % or more of the total amount of cells.

Aggregate formation is disadvantageous, because for example due to the heterogeneity in metabolic profiles of the cells within the cell aggregates, process control is more difficult.

Therefore, it is the object of the invention to provide a perfusion process in which cell aggregation is diminished.

This object is achieved by the invention, by a perfusion process wherein as a cell retention device a filter module comprising hollow fibers is used and wherein the flow within the filter module is an alternating tangential flow.

Surprisingly, the process of the invention diminishes cell aggregation. Furthermore, the process of the invention also leads to higher cell densities than when other cell retention devices in combination with other flow techniques are used.

Perfusion culturing includes, but is not limited to continuous flow and semi-continuous flow, for example step-wise flow or staggered flow.

With the term 'hollow fiber' is meant a tubular membrane. The internal diameter of the tube is preferably between 0.3 and 6.0 mm more preferably between 0.5 and 3.0 mm, most preferably between 0.5 and 2.0 mm. Preferably, the mesh size in the membrane is chosen such that the size of the pores in the mesh is close to the diameter of the cells, ensuring a high retention of cells while cell debris can pass the filter. Preferably, the meshsize is between 3-30 µm

Filter modules comprising hollow fibers are commercially available from for example Amersham.

With 'alternating tangential flow within the filter module' is meant that there is one flow in the same direction as the membrane surfaces of the hollow fibers,

which flow is going back and forth and that there is another flow in a direction substantially perpendicular to said filter surface. Tangential flow can be achieved according to methods known to the person skilled in the art. For example, in US 6,544,424 it is described that alternating tangential flow can be achieved using one pump to circulate the cell culture over a filter module comprising hollow fibers and another pump to remove the liquid comprising less cells than prior to the filter separation.

In the process of the invention, any type of cell culture medium suitable for the culturing of cells can in principle be used. Guidelines for choosing a cell culture medium and cell culture conditions are well known and are for instance provided in Chapter 8 and 9 of Freshney, R. I. Culture of animal cells (a manual of basic techniques), 4th edition 2000, Wiley-Liss and in Doyle, A., Griffiths, J. B., Newell, D. G. Cell &Tissue culture: Laboratory Procedures 1993, John Wiley & Sons.

Generally, a cell culture medium for mammalian cells comprises salts, amino acids, vitamins, lipids, detergents, buffers, growth factors, hormones, cytokines, trace elements and carbohydrates. Examples of salts include magnesium salts, for example $MgCl.6H_2O$, $MgSO_4$ and $MgSO_4.7H_2O$ iron salts, for example $FeSO_4.7H_2O$, potassium salts, for example KH_2PO_4, KCl; sodium salts, for example NaH_2PO_4, Na_2HPO_4 and calcium salts, for example $CaCl_2.2H_2O$. Examples of amino acids are all 20 known proteinogenic amino acids, for example hystidine, glutamine, threonine, serine, methionine. Examples of vitamins include: ascorbate, biotin, choline.Cl, myo-inositol, D-panthothenate, riboflavin. Examples of lipids include: fatty acids, for example linoleic acid and oleic acid; soy peptone and ethanol amine. Examples of detergents include Tween 80 and Pluronic F68. An example of a buffer is HEPES. Examples of growth factors/hormones/cytokines include IGF, hydrocortisone and (recombinant) insulin. Examples of trace elements are known to the person skilled in the art and include Zn, Mg and Se. Examples of carbohydrates include glucose, fructose, galactose and pyruvate.

The pH, temperature, dissolved oxygen concentration and osmolarity of the cell culture medium are in principle not critical and depend on the type of cell chosen. Preferably, the pH, temperature, dissolved oxygen concentration and osmolarity are chosen such that it is optimal for the growth and productivity of the cells. The person skilled in the art knows how to find the optimal pH, temperature, dissolved oxygen concentration and osmolarity for the perfusion culturing. Usually, the optimal pH is between 6.6 and 7.6, the optimal temperature between 30 and 39°C, the optimal osmolarity between 260 and 400mOsm/kg.

Aggregating cells are cells that form aggregates of at least 5 cells, the aggregates comprising in total at least 5 % of the total amount of cells. Preferably, the aggregates are at least 6, more preferably at least 7, even more preferably at least 8, even more preferably at least 9 cells. Preferably, the aggregates comprise in total at least 7%, more preferably at least 10 %, most preferably at least 15% of the total amount of cells.

Examples of aggregating cells include: some clones of mammalian cells, for example CHO (Chinese Hamster Ovary) cells, hybridomas, BHK (Baby Hamster Kidney) cells, myeloma cells, human cells, for example HEK-293 cells, human lymphoblastoid cells, PER.C6™ cells, mouse cells, for example NS0, certain clones of philamentus fungi, for example *Aspergillus niger, Aspergillus oryzae, Trichoderma reesei, Penicillium chrysogenum*, certain clones of yeasts, for example *Saccharomyces cerevisiae, Phaffia rhodozyma, Pichia pastoris or yeast cells from the genus, Hansenula*, certain clones of animal cells, bacterial cells, for example *Escherichia coli, Bacillus sp, for example B. licheniformis, B. subtilis, B. amyloliquefaciens, B. alkalophilus, Streptomyces sp., Corynebacterium glutamicum, Pseudomonas sp.*

Preferably, aggregating mammalian cells or yeast cells are used, more preferably mammalian cells, even more preferably CHO, NS0, PER.C6™ cells, most preferably PER.C6™ cells.

Cell aggregation may for example be determined under a microscope.

The rate of addition of cell culture medium (the inflow rate) is in principle not critical. In one embodiment of the invention, the cell culture medium is added at an inflow rate according to the following formula 1:

$$\text{Inflow rate} = \text{SIR} * \text{total cell culture volume} * \text{viable cell density}/10^6 \quad (1)$$

, wherein the inflow rate is expressed in liters per day, wherein the SIR is the specific inflow rate, i.e. the rate in which the cell culture medium is fed to the cell culture expressed in nl per viable cell per day and wherein the viable cell density is the number of viable cells expressed in 10^6 cells/ml. The rate of addition of the cell culture medium influences the viability and the density of the cells.

The number of viable cells can be determined by the person skilled in the art, for example via the trypan blue exclusion method. The specific inflow rate is preferably chosen between 0.01 and 0.3 nl/cell/day, more preferably between 0.01 and 0.2 nl/cell/day.

It may be of advantage to control the inflow rate using other factors, for example by using the level of glucose or oxygen as variables. For example, for PER.C6 ™ cells the glucose inflow rate is preferably chosen between 3 and 20 mmoles/l, more preferably between 5 and 15 mmoles/l.

A person skilled in the art knows how to determine the outflow rate. The outflow rate of the liquid is determined by the inflow rate and is generally chosen at an equal rate.

In another embodiment of the invention, biomass (i.e. cells in cell culture) is removed at least once from the cell culture and additional cell culture medium is added to the cell culture. Biomass removal may lead to higher cell densities. Biomass may be removed continuously or step-wise.

In the step-wise approach, biomass is removed continuously for a defined time period. If a step-wise approach is used, biomass removal is preferably started after the cells have reached a steady state. Biomass removal is preferably stopped after the cells have reached a new steady state.

If a step-wise approach is used, preferably between 2 and 40 % of the working volume per day, more preferably between 5 and 30% of the working volume per day, even more preferably between 10 and 25% of the working volume per day of the biomass is removed per biomass removal step. With 'working volume' is meant the total volume of the cell culture.

With biomass removal step is meant the time from the start to the stop of the biomass removal. Preferably, at least two biomass removal steps are employed. If more than one biomass removal step is employed, preferably, the amount of biomass removed in a later biomass removal step is less than in a former biomass removal step.

If a continuous approach is used, the biomass is removed continuously until the end of the cell culturing. Preferably, the continuous removal of biomass is started after the cells have reached a steady state. Preferably, biomass is removed at between 2 and 40 % of the working volume per day, more preferably between 3 and 30% of the working volume per day, even more preferably between 4 and 15% of the working volume per day.

The addition of the additional cell culture medium is used to compensate for the biomass removal. The feed wherein additional cell culture medium is added to the cell culture may be merged into the inflow feed, but may also be added in a separate feed. The person skilled in the art is aware how much additional cell culture medium is needed to compensate for the biomass removal. Generally, the rate

of addition of the additional cell culture medium to the cell culture will be the same as the biomass removal rate.

In yet another embodiment of the invention, a biological substance is produced by the cells. The biological substances that can suitably be produced in the perfusion culturing by the cell are in principle all biological substances that can be produced by mammalian and yeast cells, for example therapeutic and diagnostic proteins, for example monoclonal antibodies, growth factors and enzymes, DNAs, which for example might be used in gene therapy, vaccines, hormones etc.

In the perfusion culturing process of the invention, the outflow of liquid will comprise less cells and also more of the biological substance than prior to separation.

Preferably, the process according to the invention is used for the production of a biopharmaceutical product, which is a biological substance with a medical application. Examples of biopharmaceutical products are as follows (with examples of brand names of the corresponding biopharmaceutical product between brackets): Tenecteplase (TN Kase™), (recombinant) antihemophilic factor (ReFacto™); lymphoblastoid Interferon α-n1 (Wellferon™), (recombinant) Coagulation factor (NovoSeven™), Etanercept, (Enbrel™), Trastuzumab (Herceptin™), Infliximab (Remicade™), Basiliximab (Simulect™), Daclizumab (Zenapaz™), (recombinant) Coagulation factor IX (Benefix™), erythropoietin alpha (Epogen®), G-CSF (Neupogen®Filgrastim), Interferon alpha-2b (Infergen®), recombinant insulin (Humulin®), Interferon beta 1a (Avonex®), Factor VIII (KoGENate®), Glucocerebrosidase (Cerezyme™), Interferon beta 1b (Betaseron®), TNF alpha receptor (Enbrel®), Follicle stimulating hormone (Gonal-F®), Mab abcixmab (Synagis®, ReoPro®), Mab ritiximab (Rituxan®), tissue plasminogen activator (Activase ®, Actilyase®), human growth hormone (Protropin®, Norditropin®, GenoTropin™). Examples of DNAs with a possible medical application are gene therapeutic plasmid DNAs. Some gene therapeutic DNAs are presently tested in clinical trials for their medical application. Examples of vaccines are live, oral, tetravalent Rotavirus vaccine (RotaShield™), rabies vaccine (RanAvert™), Hepatitis B vaccin (RECOMBIVAX HB®, Engerix®) and inactivated hepatitis A vaccine (VAQTA™).

The biological substance in the outflow may be further purified in so-called downstream processing. Downstream processing usually comprises several purification steps in varying combinations and order. Examples of purification steps in the downstream processing are separation steps (e.g. by affinity chromatography

and/or ion exchange chromatography), steps for the concentration of the biological substance (e.g. by ultrafiltration or diafiltration), steps to exchange buffers and/or steps to remove or inactivate viruses (e.g. by virusfiltration, pH shift or solvent detergent treatment).

The invention will now be elucidated by way of the following examples, without however being limited thereto.

Example: Determination of the number of viable cells:

The number of viable cells was determined as follows: An amount of cells stained with trypan blue was transferred to a fuchs Rosenthal haemacytometer. The chamber of the haemacytometer was placed under a microscope and an appropriate number of boxes was counted. The viable cell density was calculated using the following formula:

$$\text{Viable cell density } (X10^5 \text{ cells/ml}) = (A+B) \times {}^{E}/_{320} \qquad (2)$$

Wherein
A = number of non-stained cells in square A
B = number of non-stained cells in square B
E = dilution factor

On the Cell Culture Engineering conference of 8-12 March in Cancun, Mexico the following poster is presented:

Process Optimisation of the Human Cell Line PER.C^{lm} for the production of Biopharmaceticals
John Crowley, Maike Wubben, Edith Olthof, Johanne Coté, Rodney Gagne, José M. Coco Martin. DSM Biologics, Research & Development, P.O Box 454, 9700 AL Groningen, Netherlands.

Introduction

A number of expression platforms now exist for the production of biopharmaceuticals. Most of the new products must choose a mammalian system due in main part to the glycosylation machinery which these cells contain and others lack. However to date the cell mass and resulting productivity of these cells is a factor of 10 –100 times less than a corresponding microbial system if these cells had the machinery

to make such products.

The PER.C6™ cell line is a human cell line that possess a number of features that make it favourable for the production of biopharmaceuticals. In this study, the development of the three main modes of operation (batch, fed-batch and continuous perfusion) for the PER.C6™ cell line were developed. The fed-batch process is based on the stoichiometric feeding of a balanced nutrient concentrate matched to the metabolic requirements of a model antibody producing PER.C6™ cell line.

A perfusion setup involves the separation of various components of the fermenter broth so that cells are retained, harvest is captured and medium refreshment occurs. The performance of a spinfilter, an acoustic device and an alternating tangential flow filter (ATF) within a developed high cell density continuous perfusion the PER.C6™ cell line was assessed.

Materials & Methods

Cell line and maintenance : A PER.C6™ cell line was used in this study that produces a human IgG. Cells were maintained in a serum free commercial medium. The PER.C6™ cell line is human embroyonic cel-line immortalised with adenovirus type-5 (ad5) E1 gene using a phosphoglyceratekinase promoter.

Bioreactor Set-up: A 4L working volume reactor (Applikon, Netherlands) was used during this study. A Braun DCU3 controller (B.Braun, Germany) was used to operate the process at defined setpoints. Temperature was maintained at 37oC by a hot/cold finger. Dissolved oxygen concentration was controlled at 50% of air saturation by automatic adjustment of inlet gas composition through the headspace and intermittent sparging through a microporous sparger.

Cell Retention: Cells were retained in the reactor using two different devices. A Biosep 10L acoustic filter chamber and associated ASP991 controller (Applisens, Netherlands, a spinfilter basket (10um pore size) and an alternating tangential flow filter (ATF) were assessed. To maintain a constant culture volume a level sensor control loop was in operation.

Analytical methods : A cell count from the bioreactor was performed using the trypan

blue exclusion method. Antibody concentration was determined by a analytical protein A column using a HPLC.

Results

Batch and Fed-Batch Cultures

Results obtained with the above materials and methods are shown in the figures.

Legends to the figures:

Figure 1: IgG concentration versus culture time (days) for a standard batch and developed fed-batch performed with the PER.C6™ cell line.

Figure 2: Antibody concentration versus culture time (days) for five 7L and one 70L batch fermentation performed with the PER.C6™ cell line.

Figure 3: Comparison of PER.C6™ cells in batch culture and a culture with a daily medium exchange.

Figure 4: Viable cell density ($x10^6$ cells/ml) versus culture time (days) for two different continuous perfusion fermentations of a Mab producing PER.C6™ clone differing specific perfusion rates (nl/cell/day) using a spinfilter separation device.

Figure 5: Growth of PER.C6™ cells in a continuous perfusion system with an acoustic device as a cell retention system.

Figure 6: Growth of PER.C6™ cells in a continuous perfusion system with an Alternating Tangential Flow filter as a cell retention system.

Figure 7: Productivity versus culture time (days) for two different continuous perfusion fermentations of a Mab producing PER.C6™ clone differing specific perfusion rates (nl/cell/day; closed circle ; 0.4 nl/cell/day) using a spinfilter separation device.

Figure 8: Productivity of PER.C6™ cells in a continuous perfusion system with an acoustic device as a cell retention system

Figure 9: Productivityof PER.C6™ cells in a continuous perfusion system with an Alternating Tangential Flow (ATF) filter as a cell retention system.

Figure 10: Photograph of a cell density and supernatant produced using PER.C6 ™ cells.

Figure 11: Cell density ($x10^6$ cells/ml) versus culture time (days) for PER.C6™ cells grown in a continuous perfusion system using a spin basket separation device.

Figure 12: Volumteric production rate (g/L/day) versus culture time (days) for PER.C6™ cells grown in a continuous perfusion system using a spin basket separation device.

Figure 13: Cell density ($x10^6$ cells/ml) versus culture time (days) for PER.C6™ cells grown in a continuous perfusioin system using an acoustic retention separation device.

Figure 14: Volumteric production rate (g/L/day) versus culture time (days) for PER.C6™ cells grown in a continuous perfusion system using an acoustic retention separation device.

Figure 15: Viable cell density ($x10^6$ cells/ml) versus culture time (days) for PER.C6™ cells grown in a continuous perfusioin system using an alternating tangential flow (ATF) filtration device.

Figure 16: Volumetric production rate (g/L/day) versus culture time (days) for PER.C6™ cells grown in a continuous perfusion system using an alternating tangential flow (ATF) filtration device.

Figure 17: Culture time (days) versus flow (L/day) and specific perfusion rate (SPR in nl/cell/day) for PER.C6™ cells cultured using a perfusion process.

Figure 18: Viable cell density (Figure 18: Culture time (days) versus flow (L/day and specific perfusion rate (nl/cell/day) for PER.C6™ cells cultured using a perfusion process.

Summary

Table 1:

Process	Max. Viable Cell Density (10^6 cells/mL)	Productivity	Yield Improvement Factor[1]
Batch	8-10	0.5 g/L	1
Fed-Batch	8-10	1.2 g/L	2.4
Continuous Perfusion			
Process 1	20-30	0.1-0.2 g/L/day	2.8-5.6
Process 2	20	0.6 g/L/day	16.8
Process 3	100	0.9 g/L/day	25.2

1 Normalized to a 14 day fed-batch process. Batch process is set as 1.

Conclusions:

- The initial performance of Per.C6™ in a fed-batch mode is already equivalent to other cell lines (1.2g/L).
- Early continuous perfusion experiments shows significant potential to achieve very high densities and product concentrations ($100x10^6$ cells/mL and 0.9 g/L/day).

Also on the same conference the following abstract is published:

Process optimization of the human cell line PER.C6™ for the production of biopharmaceuticals

John Crowley, Maike Wubben, Edith Olthof, Jose M. Coco Martin.

DSM Biologics, Research & Development, P.O Box 424, 9700 AL Groningen, Netherlands.

A number of expression platforms now exist for the production of biopharmaceuticals. PER.C6™ is a human cell line that possess a number of features

that make it favourable for the production of biopharmaceuticals. The factors involved in choosing such a platform will be discussed.

Having chosen an expression platform, the three main methods of production typically batch, fed-batch and continuous perfusion were developed for a model antibody producing PER.C6™ cell. The mode of operation is typically chosen on the basis of product concentration, quality and stability, scalability, simplicity, time and cost. In this presentation, the development of all three operation modes for the PER.C6™ will be presented. The fed-batch process is based on the stoichiometric feeding of a balanced nutrient concentrate matched to the metabolic requirements of a model antibody producing PER.C6™ cell yielding equivalent productivity to other cell lines in a short time period.

A perfusion setup involves the separation of various components of the fermenter broth so that cells are retained, harvest is captured and medium refreshment occurs. The performance of a spinfilter, alternating tangential flow (ATF) and acoustic separation devices within a high cell density process using the PER.C6™ cell line will be presented. In conclusion, the early development of a continuous perfusion process for an antibody producing PER.C6™ cell will be presented yielding significantly high cell densities and volumetric production rates.

Process Example 2

This example relates to a process for culturing of PER.C6™ cells by perfusion as defined previously.

Equipment: B.Braun fermenter control unit (Braun, Germany), 7L Braun vessel and headplate with associated pH, dissolved oxygen (DO) and level sensor probes (Braun, Germany), ATF-4 control unit and housing with associate hollow fibre membrane module (Refine Technology, USA).

Filter

Filter model: CFP-2-E-8SIP
Type: 0.2 micron
Area: 4600cm^2
Amersham Bioscience

Working volume

Setpoint: 4.1 L

Range: 3.8 – 4.7 L

ATF settings

Parameter	Setpoint	Range
Pressure rising setpoint (psi)	Variable	2 - 4
Pressure rising flow (L/min)	3.2	2.5 – 4.0
Exhaust flow (L/min)	3.2	2.5 – 4.0
Exhaust time (s)	Variable	3 - 8
Pre-pressure (psi)	Variable	5 - 9

Bleed rate

No biomass removal was applied to this process.

Additions

At day 16 two drops of antifoam (BC Antifoam Simethicone C100F; Basildon Chemicals Company Limited, USA), were added to the fermenteer in order to decrease the foam layer.

Materials: 6 mmole L-glutamine (Gibco) in VPRO mammalian medium (JRH, USA). 12% Na_2CO_3 is used to control the pH.

Cell Lines and Culture Conditions

A PER.C6™ cell line expressing a model IgG was investigated in this study. The PER.C6™ cell line is generated from retina-derived primary human cells. The PER.C6™ cell line is able to generate complete human monoclonal antibodies (including the glycans) (ref 1, ref 2).

Cells were cultured in shaking Erlenymer flasks at 110 rpm and 36.5°C . The headspace of these flasks was controlled using a mixture of 5% CO_2/Air.

Ref 1: Jones, D. H., van Berkel, P. H. C., Logtenberg, T. and Bout, A., 2002, 'PER.C6 cell line for human antibody production.', Gen. 22, ed. May 15.

Ref 2: Jones, D. et al., 2003, 'High-level expression of recombinant IgG in the human

cell line PER.C6.', Biotechnol. Prog. 19, 163-168.

Operation of Fermenter

Cells were cultured in a fermenter where dissolved oxygen tension, pH, temperature and agitation rate were controlled as detailed below.

Parameter	Setpoint	Range
Temperature	36.5°C	35.5 – 37.5
pH	> 6.7	7.5 –6.7, Active pH control using 12% Na_2CO_3 if pH<6.7
DO	50%	40 –60%
Agitation	100 – 300	Staged increase as viable cell density (VCD) increases;

VCD ($x10^6$ cells/ml)	Agitation (rpm)
0.3 – 10	120
10 –30	150
30 –50	170
50 –80	200
80 – 100	230
100 –120	260
>120	300

Process Description : Cells are innoculated in a fermenter with an innoculation viable cell density range of 0.2 – 0.5 x 10^6cells/ml and a setpoint of 0.3 x 10^6 cells/ml. Perfusion is begun when the viable cell density > 2 x 10^6 cells /ml or at day 5 of the culture whichever is achieved first.

The perfusion rate is dependent on the cell density of the culture and the rates used are described in the table below. Both the flow rate and the dilution rate are adjusted as the cell density in the fermenter increases.

Perfusion rates utilized for culture of PER.C6™ cells

Viable cell density ($\times 10^6$ cells/ml)	Specific perfusion rate (ml/cell/day)	Setpoint of specific perfusion rate (ml/cell/day)
Day 1 of perfusion	0.15 – 0.25	0.2
3 – 50	0.03 – 0.06	0.04
50 –80	0.025 – 0.035	0.03
>80	0.01 – 0.03	0.02

The actual data from this example (amongst others flowratres and specific perfusion rates used in this example) are shown in Table A below

Table A. Actual data from process example 2

Time	Flow rate (FR)	Dilution rate (D)	Specific perfusion rate (SPR)	Viable cell count (VCC)	Viability	Product concentration	Specific production rate of IgG1	Volumetric production rate
day	L/day	Working volume/day	nL/cell/day	10^6/mL	%	g/L	pg/(cell day)	g/L/day
0	0.00	0.00	0.00	0.6	90	0.012	NA	NA
1	0.00	0.00	0.00	0.3	77	0.008	NA	0.000
2	0.00	0.00	0.00	0.3	73	0.008	0.0	0.000
3	0.00	0.00	0.00	0.5	80	0.013	12.1	0.000
4	0.00	0.00	0.00	0.9	87	0.019	9.3	0.000
5	0.00	0.00	0.00	1.4	92	0.033	12.0	0.000
6	2.39	0.52	0.20	2.6	95	0.035	5.5	0.009
7	1.06	0.24	0.05	4.9	95	0.054	9.5	0.017
8	2.70	0.57	0.08	7.3	97	0.073	7.2	0.026
9	2.60	0.57	0.05	12.3	97	0.067	3.5	0.040
10	4.29	0.95	0.05	18.6	97	0.115	7.8	0.069
11	5.40	1.20	0.04	26.9	97	0.140	7.0	0.137
12	6.80	1.48	0.05	31.8	96	0.127	5.6	0.179
13	7.39	1.68	0.04	41.4	99	0.129	5.6	0.202
14	8.28	1.88	0.04	44.3	98	0.139	5.8	0.238
15	10.26	2.33	0.03	68.3	98	0.116	4.4	0.269
16	10.70	2.43	0.03	86.1	99	0.151	4.6	0.318
17	12.10	2.63	0.03	80.3	98	0.163	4.9	0.397
18	11.83	2.57	0.02	112.3	98	0.292	7.6	0.592
19	12.50	2.78	0.02	123.0	99	0.291	6.6	0.780
20	12.09	2.57	0.02	126.0	99	0.293	6.3	0.781
21	11.91	2.59	0.02	135.0	98	0.332	6.5	0.806
22	13.70	2.98	0.02	127.5	97	0.395	8.2	1.012
23	10.00	2.17	0.02	128.6	95	0.470	9.3	1.114

Figure 17 shows the actual flowrates and specific perfusion rates used in process example 2.

Figure 18 shows the cell density achieved using the procedure described in this process example 2.

5

CLAIMS

1. Process for the culturing of cells by continuous perfusion culturing of a cell culture comprising cell culture medium and cells that form aggregates of 5 cells or more and the aggregates comprise in total 5% or more of the total amount of cells, wherein cell culture medium is added to the cell culture and wherein the cell culture is circulated over a cell retention device resulting in an outflow of liquid comprising less cells than the cell culture, characterized in that as a cell retention device a filter module comprising hollow fibers is used and in that the flow within the filter module is an alternating tangential flow.
2. Process according to claim 1, wherein the alternating tangential flow is achieved using one pump to circulate the cell culture over a filter module comprising hollow fibers and using another pump to remove the liquid comprising less cells than the cell culture.
3. Process according to claim 1 or claim 2, wherein the cells are mammalian cells.
4. Process according to claim 3, wherein the mammalian cells are PER.C6™ cells.
5. Process according to any one of claims 1-4, wherein the cell culture medium is added at an inflow rate calculated according to formula 1:

   Inflow rate = SIR*total cell culture volume*viable cell density/10^6 (1)

   , wherein the inflow rate is expressed in liters per day, wherein the SIR is the specific inflow rate, i.e. the rate in which the cell culture medium is fed to the cell culture expressed in ml per viable cell per day and wherein the viable cell density is the number of viable cells expressed in 10^6 cells/ml.
6. Process according to claim 5, wherein the specific inflow rate is between 0.01 and 0.3 ml/cell/day.
7. Process according to any one of claims 1-6, wherein biomass is removed at least once from the cell culture and wherein additional cell culture medium is added to the cell culture.
8. Process according to claim 7, wherein the biomass removal is started after the cells have reached a steady state.
9. Process according to claim 7 or claim 8, wherein the rate of biomass removal is between 2 and 40% of the total volume of the cell culture.
10. Process according to any one of claims 1-9, wherein the cells produce a biological substance.

11. Process according to claim 10, wherein the biological substance is a monoclonal antibody.

12. Process according to claim 10 or claim 11, wherein the biological substance is further purified in downstream processing.

ABSTRACT

The invention relates to a process for the culturing of cells by continuous perfusion culturing of a cell culture comprising cell culture medium and cells that form aggregates of 5 cells or more and the aggregates comprise in total 5% or more of the total amount of cells, wherein cell culture medium is added to the cell culture and wherein the cell culture is circulated over a cell retention device resulting in an outflow of liquid comprising less cells than the cell culture, wherein as a cell retention device a filter module comprising hollow fibers is used and wherein the flow within the filter module is an alternating tangential flow. The invention also relates to such a process wherein a biological substance, preferably an antibody is produced by the cells, which biological substance may be further purified in downstream processing.

Fig. 1

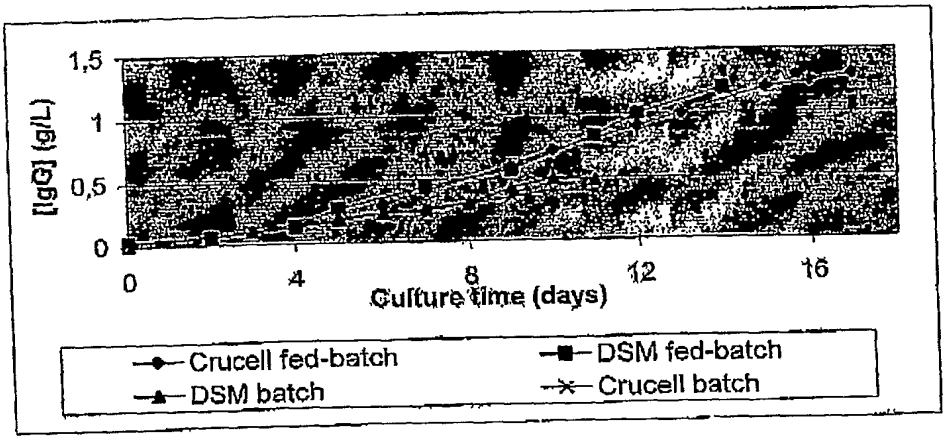

1,5
1
0,5
0
[IgG] (g/L)
0
4
8
12
16
Culture time (days)
Crucell fed-batch
DSM fed-batch
DSM batch
Crucell batch

Fig. 2

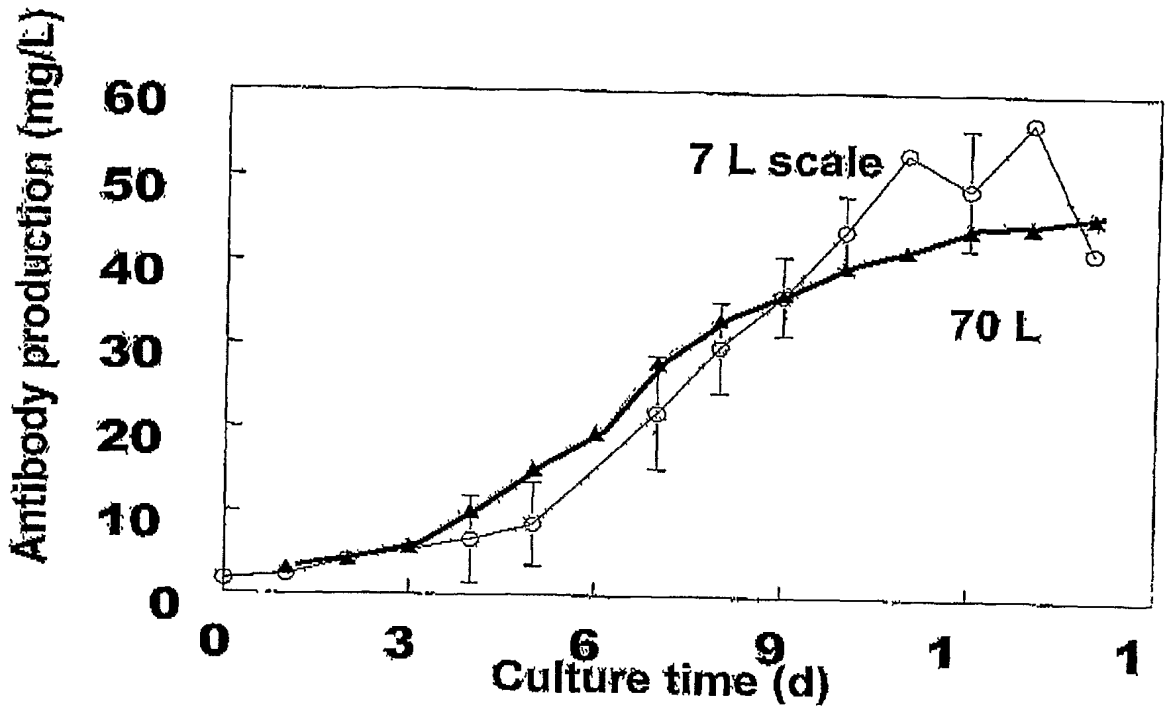

Antibody production (mg/L)
60
50
40
30
20
10
0
0
3
6
9
1
1
Culture time (d)
7 L scale
70 L

Fig. 3

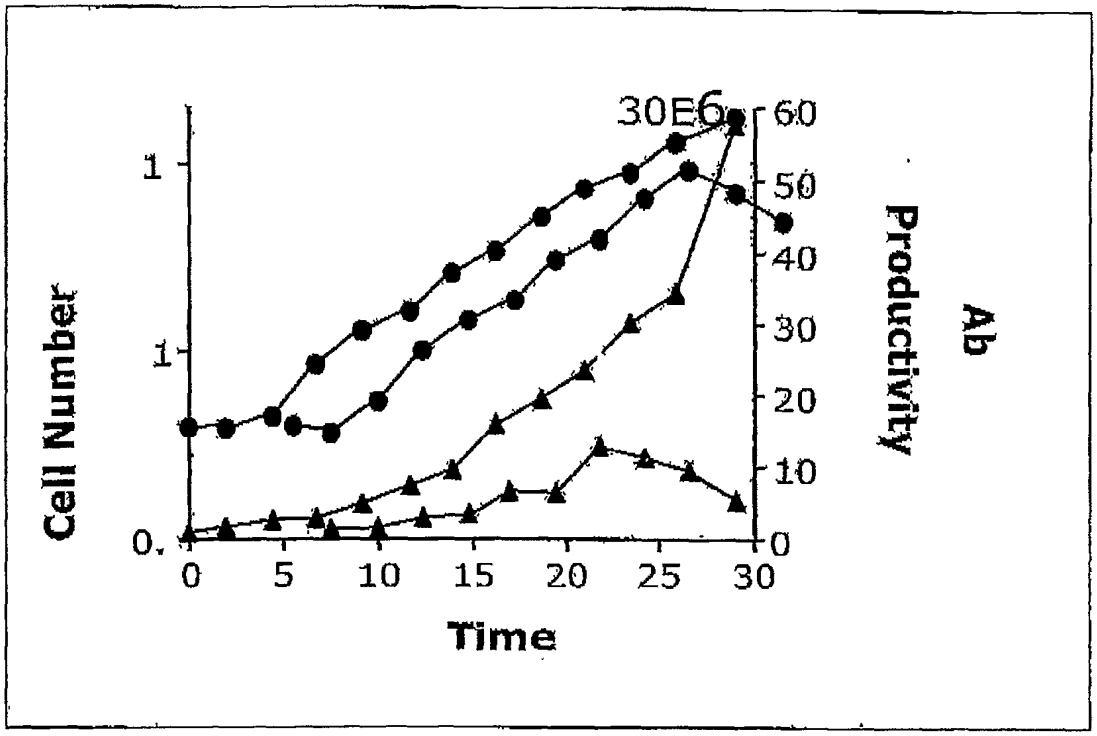

30E6
60
50
40
30
20
10
0
1
1
0.
0
5
10
15
20
25
30
Time
Cell Number
Productivity
Ab

Fig. 4

5

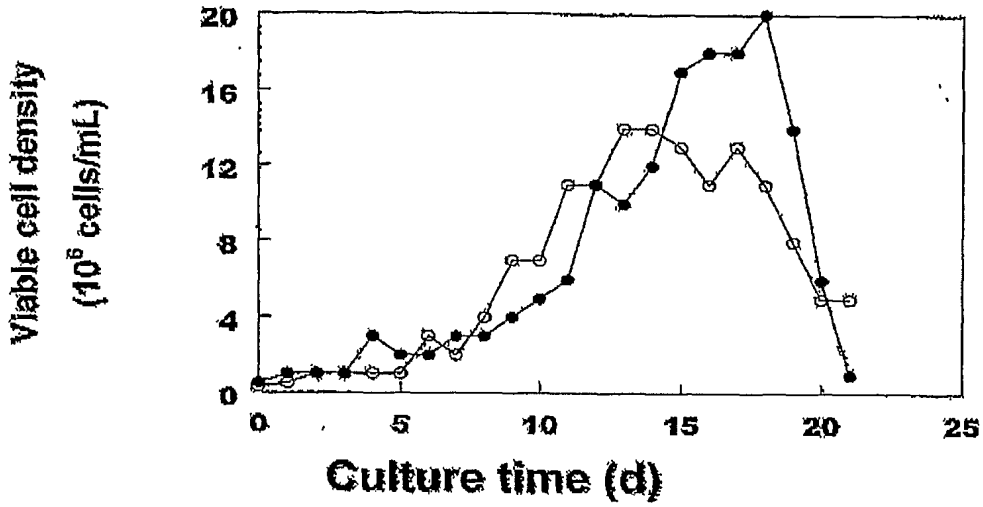

Viable cell density
(10^6 cells/mL)
20
16
12
8
4
0
0
5
10
15
20
25
Culture time (d)

Fig. 5

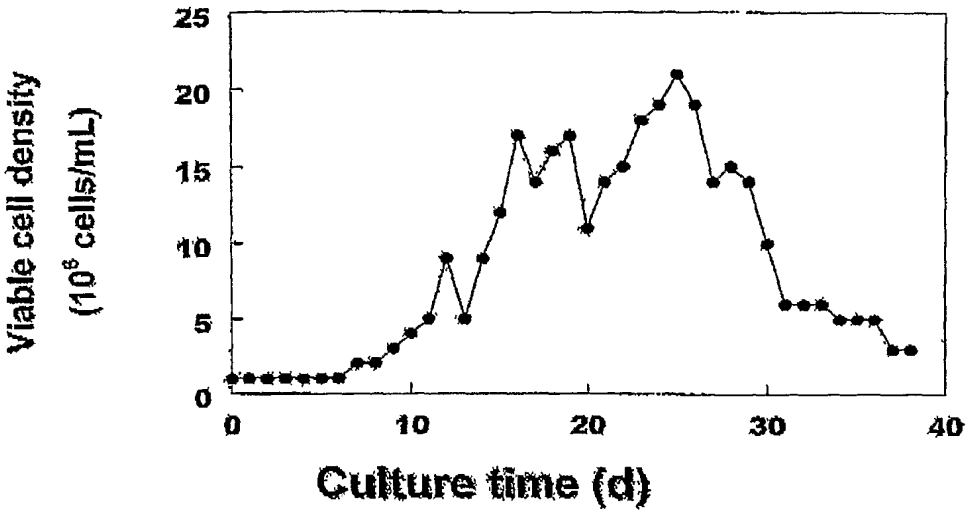

Viable cell density
(10^6 cells/mL)
25
20
15
10
5
0
0
10
20
30
40
Culture time (d)

Fig. 6

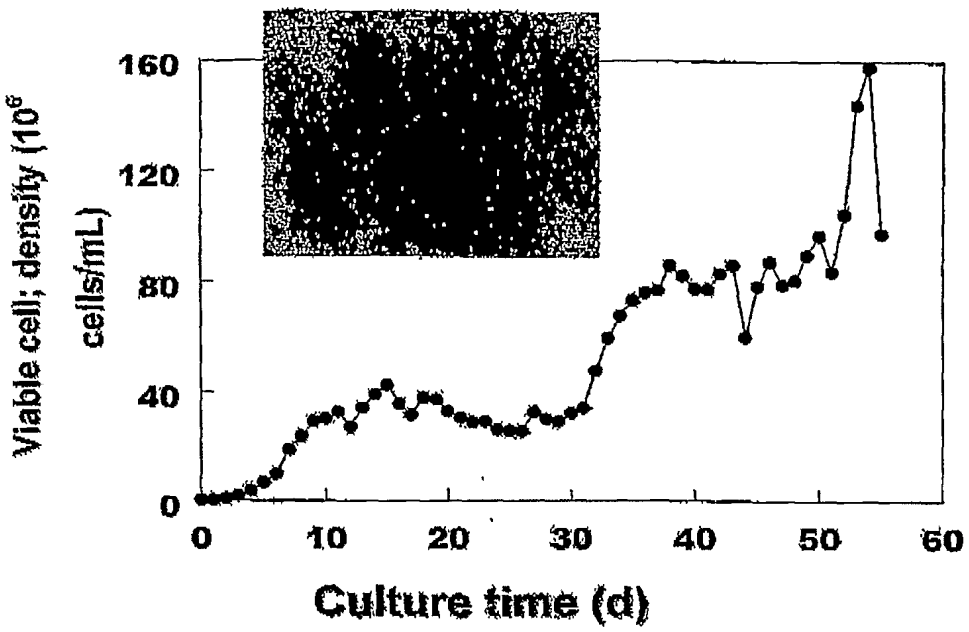

Viable cell; density (10^6 cells/mL)
160
120
80
40
0
0
10
20
30
40
50
60
Culture time (d)

Fig. 7

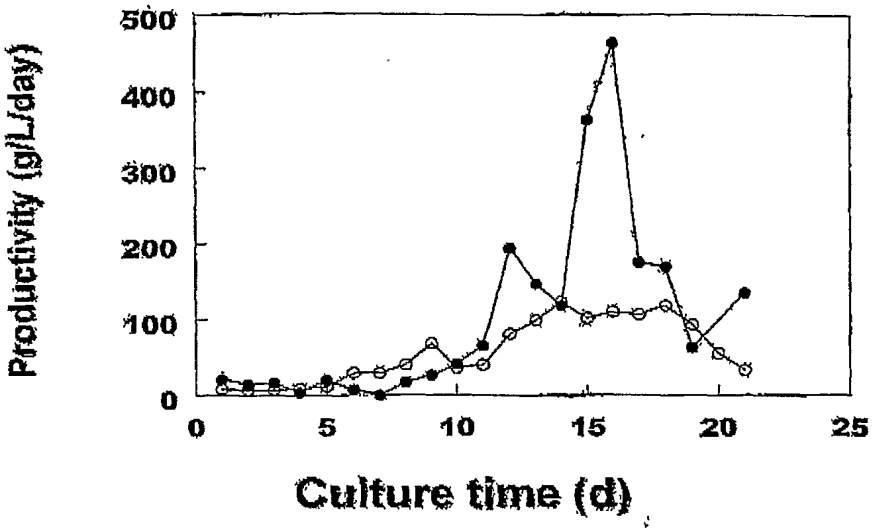

Productivity (g/L/day)
500
400
300
200
100
0
0
5
10
15
20
25
Culture time (d)

Fig. 8

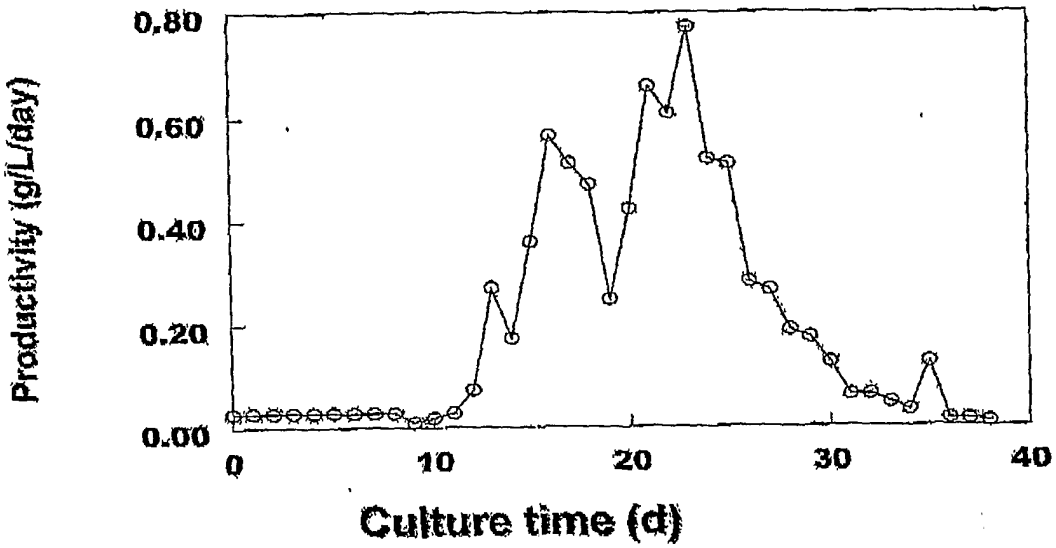

Productivity (g/L/day)
0.80
0.60
0.40
0.20
0.00
0
10
20
30
40
Culture time (d)

Fig. 9

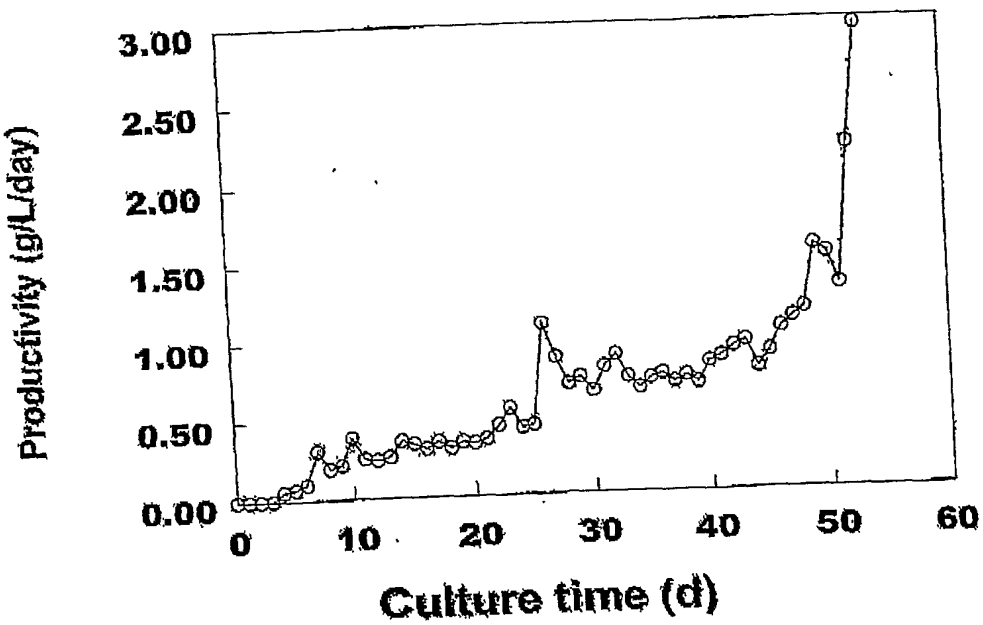

Productivity (g/L/day)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
10
20
30
40
50
60
Culture time (d)

Fig. 10

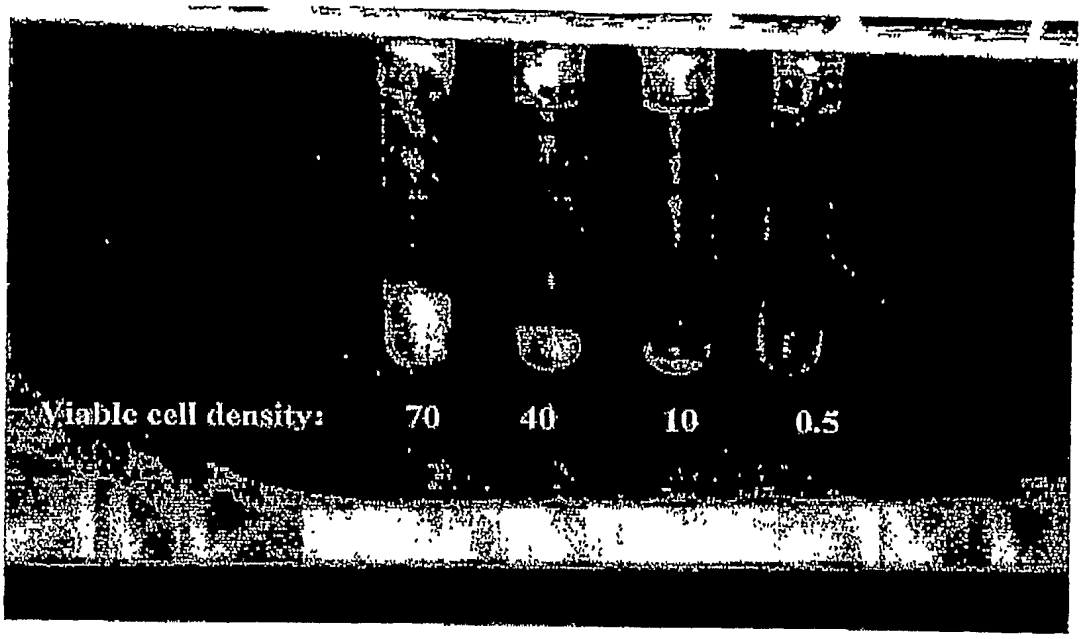
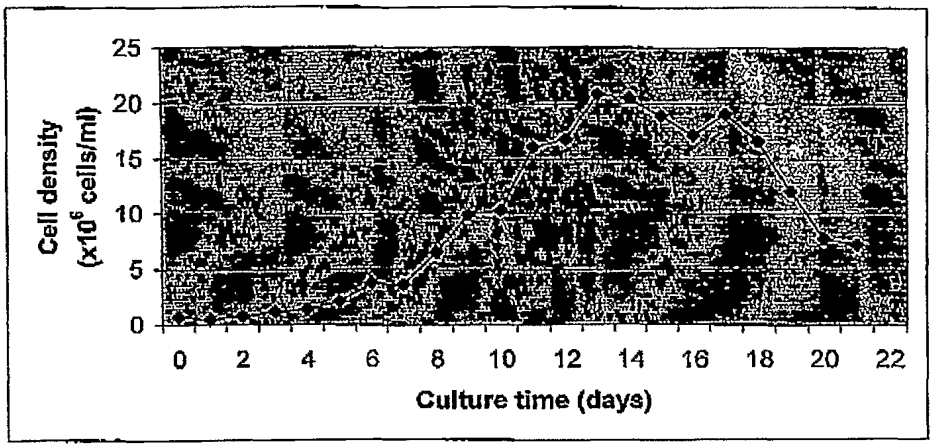


Fig. 11


Cell density
(x10^6 cells/ml)
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18
20
22
Culture time (days)

5

Fig. 12

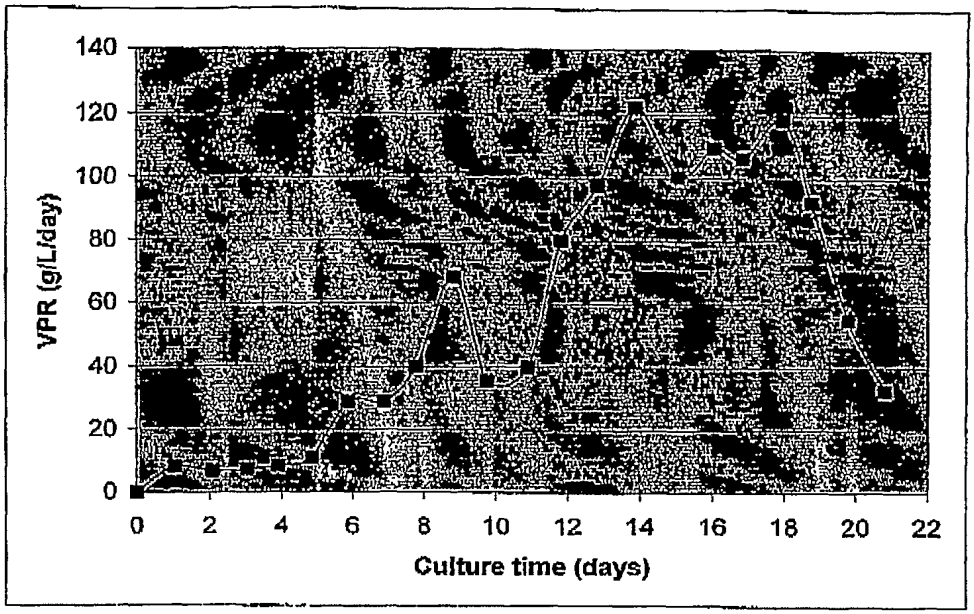

VPR (g/L/day)
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
22
Culture time (days)

Fig. 13

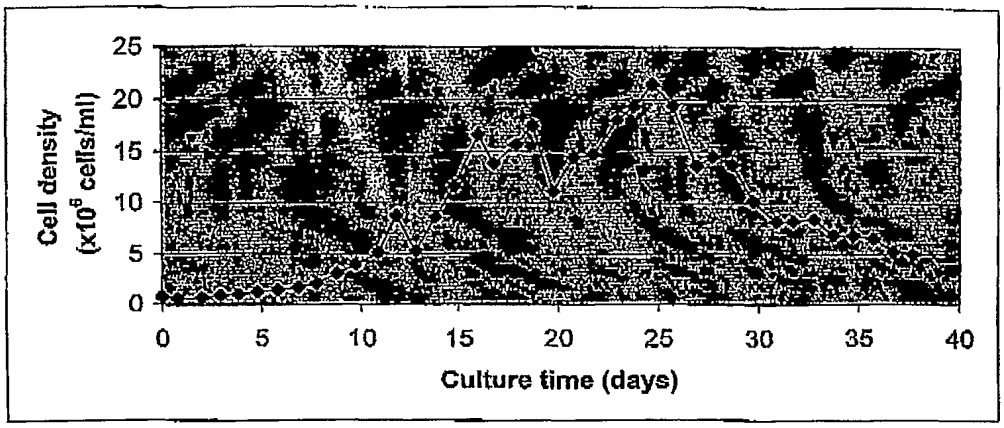
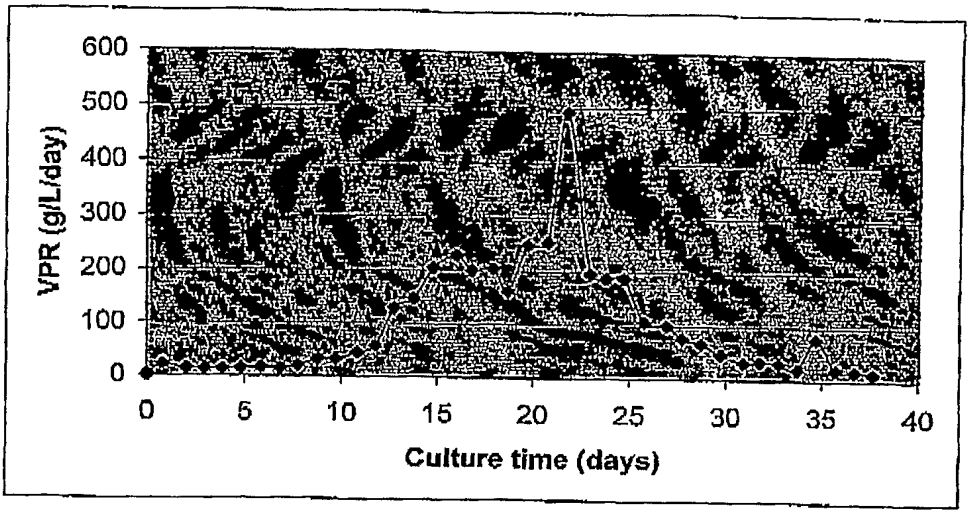

Cell density
(x10^6 cells/ml)
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
Culture time (days)

Fig. 14


VPR (g/L/day)
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
Culture time (days)

5

Fig. 15

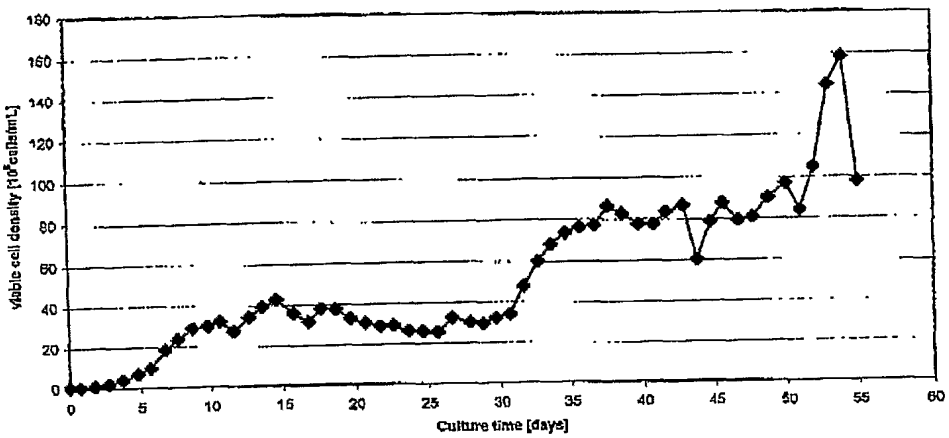
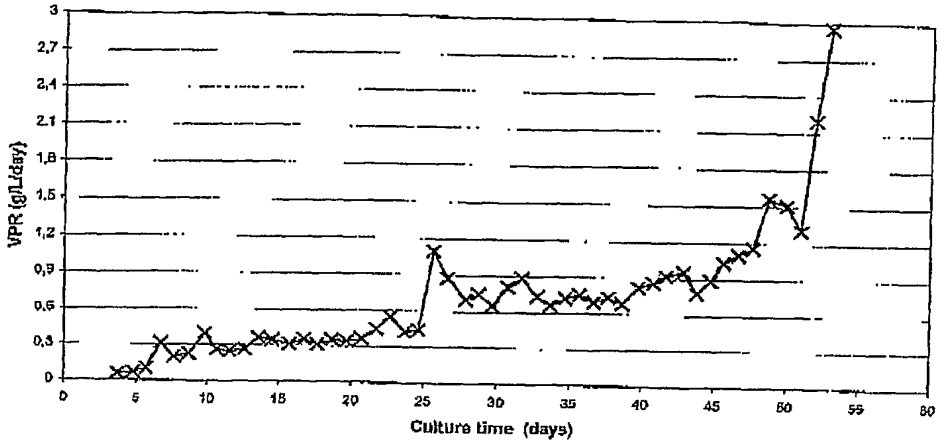

Viable cell density [10⁶cells/mL]
Culture time [days]
0
20
40
60
80
100
120
140
160
180
0
5
10
15
20
25
30
35
40
45
50
55
60

Fig. 16


VPR (g/L/day)
3
2,7
2,4
2,1
1,8
1,5
1,2
0,9
0,6
0,3
0
0
5
10
15
20
25
30
35
40
45
50
55
60
Culture time (days)

5

Fig. 17

5

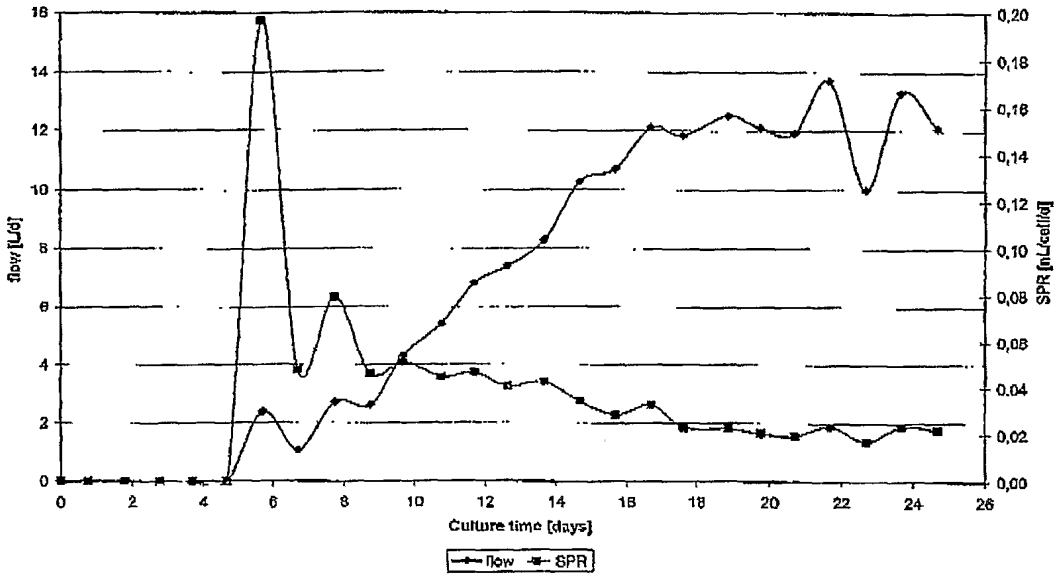
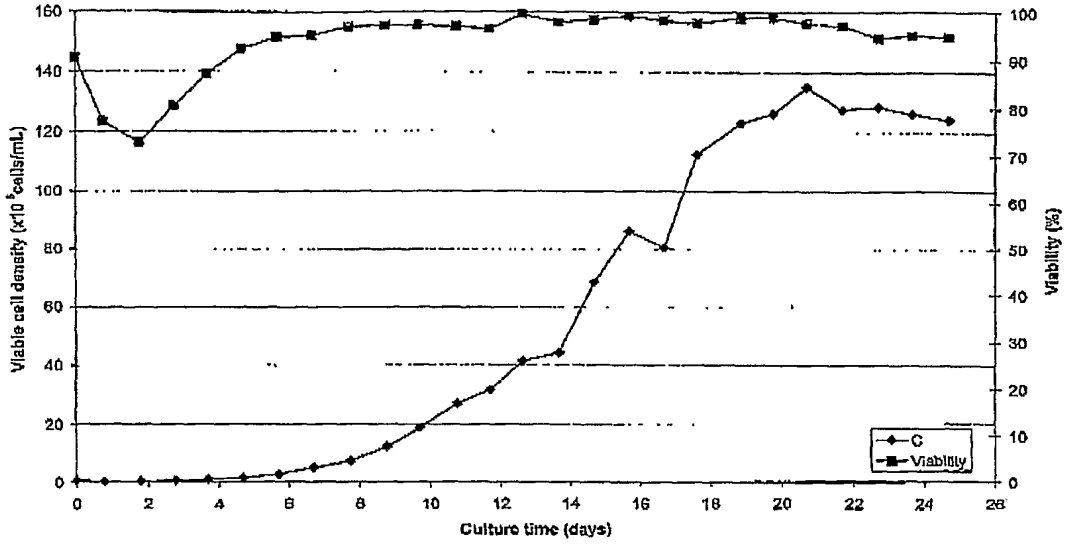

flow [L/d]
SPR [mL/cell/d]
Culture time [days]
flow
SPR
0
2
4
6
8
10
12
14
16
18
20
22
24
26
0,00
0,02
0,04
0,06
0,08
0,10
0,12
0,14
0,16
0,18
0,20

Fig. 18


Viable cell density (x10 5cells/mL)
Viability (%)
Culture time (days)
C
Viability
0
2
4
6
8
10
12
14
16
18
20
22
24
26
20
40
60
80
100
120
140
160
10
30
50
70
90